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MINIREVIEW

Pathogenesis of West Nile Virus Infection: a Balance between Virulence, Innate and Adaptive Immunity, and Viral Evasion

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West Nile virus (WNV) is a neurotropic flavivirus that has emerged globally as a significant cause of viral encephalitis. WNV is maintained in an enzootic cycle between mosquitoes and birds (reviewed in reference 75) but can also infect and cause disease in horses and other vertebrate animals. Infection of humans is associated with a febrile illness that can progress to a lethal encephalitis with symptoms including cognitive dysfunction and flaccid paralysis (30, 148, 167). Since the mid-1990s, outbreaks of WNV fever and encephalitis have occurred in regions throughout the world where WNV is endemic, including the Middle East, Europe, and Africa (43). Following its introduction into the United States in 1999, WNV rapidly disseminated across North America and more recently has been reported in Mexico, South America, and the Caribbean (45, 95, 102). Annual outbreaks of WNV fever and neuroinvasive disease occur in the United States (67), with ~19,000 diagnosed human cases between 1999 and 2005 (http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm#maps) and an estimated 750,000 undiagnosed infections in 2003 (25). Although vaccines are available for animal use, no vaccines or specific therapies for WNV are currently approved for humans.

WNV is a member of the *Flaviviridae* family of RNA viruses and is related to other important human pathogens, including dengue viruses, yellow fever viruses, and Japanese encephalitis viruses. Similar to other flaviviruses, WNV is an enveloped virus with a single-stranded, positive sense, ~11-kb RNA genome. The genome is transcribed as a single polyprotein that is cleaved by host and viral proteases into three structural and seven nonstructural proteins (32). The structural proteins include a capsid protein (C) that binds viral RNA, a premembrane (prM) protein that blocks premature viral fusion and may chaperone envelope (E)-protein folding, and an E protein that mediates viral attachment, membrane fusion, and viral assembly (131). The viral nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A NS4B, and NS5) regulate viral transcription and replication and attenuate host antiviral responses (17, 71, 87, 110, 111, 115, 132). NS1 has cofactor activity for the viral replicase and is secreted from infected cells, NS2A inhibits interferon (IFN) responses and may participate in virus assembly, and NS3 has protease, NTPase, and helicase activities (87, 113–115, 118). NS2B is a cofactor required for NS3 proteolytic activity, NS4A and NS4B modulate IFN signaling, and NS5 encodes the RNA-dependent RNA polymerase and a methyltransferase (49, 86, 115, 202).

Several molecules have been implicated in the attachment of WNV to cells in vitro, including DC-SIGN, DC-SIGN-R, and the integrin $\alpha_v\beta_3$ (37, 44). However, attachment receptors in vivo for physiologically important cell types, such as neurons, remain uncharacterized. After binding, WNV enters cells via receptor-mediated endocytosis that may involve clathrin-coated pits (36, 63). Following a pH-dependent conformational change in the E protein, the viral and endosomal membranes fuse, releasing the viral nucleocapsid into the cytoplasm (2, 23, 65). WNV is believed to replicate on endoplasmic reticulum-associated membranes to generate a negative-strand RNA intermediate that serves as a template for nascent positive-strand RNA synthesis (119). Positive-strand RNA is either packaged within progeny virions or used to translate additional viral proteins. WNV assembles and buds into the endoplasmic reticulum to form immature particles that contain the prM protein. Following transport through the trans-Golgi network, furin-mediated cleavage of prM to M generates mature, infectious virions that are released by exocytosis (51, 70).

The emergence of WNV in the western hemisphere and the corresponding increase in disease burden have been accompanied by intensive study. Epidemiological analyses indicate that the elderly and immunocompromised are at greatest risk of developing severe neurological disease (20, 35, 67, 135, 179). Experimental studies have made significant progress in dissecting the viral and host factors that determine the pathogenesis and outcome of WNV infection. This review will focus on the interactions between WNV pathogenesis and the innate and adaptive host immune responses.

PATHOGENESIS OF WNV INFECTION

Clinical manifestations. Seroprevalence studies suggest that while the majority of WNV infections are asymptomatic, approximately 20 to 30% of infected individuals develop flu-like clinical manifestations characterized as WNV fever (148, 189). In a subpopulation of individuals (approximately 1 in 150), a neuroinvasive disease develops (149, 190). The clinical features of severe WNV infection vary and include severe headache, ocular manifestations, muscle weakness, cognitive impairment,

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tremors, and a poliomyelitis-like flaccid paralysis (8, 30, 148, 167, 168). The mortality rate following neuroinvasive infection is approximately 10% (138, 148), and long-term neurological sequelae are common (>50%) (92, 166). Neuronal damage is most prevalent in the brain stem and anterior-horn neurons of the spinal cord, although in immunosuppressed individuals infection can disseminate throughout the central nervous system (CNS) (69, 94).

Viral dissemination and pathogenesis in vivo. Rodent models have provided insight into the mechanisms of WNV dissemination and pathogenesis (Table 1). Following peripheral inoculation, initial WNV replication is thought to occur in skin Langerhans dendritic cells (26). These cells migrate to and seed draining lymph nodes, resulting in a primary viremia and subsequent infection of peripheral tissues such as the spleen and kidney. By the end of the first week, WNV is largely cleared from the serum and peripheral organs and infection of the CNS is observed in a subset of immunocompetent animals. Rodents that succumb to infection develop a CNS pathology similar to that observed in human WNV cases, including infection and injury of brain stem, hippocampal, and spinal cord neurons (46, 50, 55, 140, 172, 195). WNV infection is not significantly detected in nonneuronal CNS cell populations in humans or animals. In surviving wild-type mice, WNV is cleared from all tissue compartments within 2 to 3 weeks after infection. However, persistent viral infection in the brains of $CD8^+$ T-cell (171)- or perforin-deficient mice (173) and in the brains and kidneys of infected hamsters has been reported (178, 195). Persistent infection has also been documented in a WNV-infected immunosuppressed patient in whom viremia was detected for more than 60 days (22). Although less in known about WNV pathogenesis in avian hosts, the virus has been detected by histology, reverse transcription-PCR, and virologic assays in the brains, livers, lungs, spleens, hearts, and kidneys of naturally infected crows and blue jays (59, 144, 194).

The mechanisms by which WNV and other neurotropic flaviviruses cross the blood-brain barrier (BBB) remain largely unknown, although tumor necrosis factor alpha (TNF-α)-mediated changes in endothelial cell permeability may facilitate CNS entry (186). It is likely that WNV infects the CNS at least in part via hematogenous spread, as an increased viral burden in serum correlates with earlier viral entry into the brain (47, 160). Additional mechanisms may contribute to WNV CNS infection, including (i) infection or passive transport through the endothelium or choroid plexus epithelial cells (98), (ii) infection of olfactory neurons and spread to the olfactory bulb (129), (iii) a "Trojan horse" mechanism in which the virus is transported by infected immune cells that traffic to the CNS (58), and (iv) direct axonal retrograde transport from infected peripheral neurons (78, 81). Although the precise mechanisms of WNV CNS entry in humans require additional study, changes in cytokine levels that may modulate BBB permeability and infection of blood monocytes and choroid plexus cells have been documented in animal models (58, 160, 186).

INNATE IMMUNE RESPONSES TO WNV INFECTION

Interferons. Type I (IFN-α and IFN-β), type II (IFN-γ), and type III (IFN-λ) IFNs act as important innate immune system controls of viral infections (reviewed in references 147 and 151). IFN-α/β is produced by most cell types following virus infection and induces an antiviral state by upregulating genes with direct and indirect antiviral functions. IFN-α/β also links innate and adaptive immune responses through stimulation of dendritic-cell maturation (7), by direct activation of B and T cells (105), and by preventing the death of recently activated T cells (121). Pretreatment with IFN-α/β inhibits WNV replication in vitro, but treatment after infection is much less effective (3, 41, 160). Although WNV can directly antagonize IFN-induced responses after infection (71, 114, 115), type I IFN is still required to restrict WNV replication and spread (160). Mice lacking the IFN-α/β receptor (IFN-α/βR) had uncontrolled viral replication, rapid dissemination to the CNS, and enhanced lethality. Altered viral tropism in IFN-α/β$R^{-/-}$ mice was also observed with enhanced infection in normally resistant cell populations and peripheral tissues.

IFN-γ is produced primarily by γδ T cells, $CD8^+$ T cells, and natural killer (NK) cells and limits virus infection through several mechanisms. IFN-γ restricts viral replication directly by inducing an antiviral state or indirectly by modulating the adaptive immune response through activation of myeloid cells, inducing $CD4^+$ T-cell activation and TH1-TH2 polarization, and increasing cell surface expression of major histocompatibility complex (MHC) class I molecules (34, 165). Although WNV is also resistant to the antiviral effects of IFN-γ after infection in vitro (160), in vivo IFN-γ limits early viral dissemination to the CNS: mice deficient in either IFN-γ or the IFN-γ receptor showed a greater peripheral viral burden, earlier entry into the CNS, and increased lethality (174, 185). Interestingly, no major deficits in adaptive immune responses were observed in these studies, suggesting that the dominant function of IFN-γ in controlling WNV infection is innate and antiviral. Additional experiments demonstrated a cell-specific requirement for IFN-γ, as γδ T cells utilized IFN-γ to limit WNV dissemination whereas $CD8^+$ T cells did not (174, 185).

Viral sensors and IFN-induced effector molecules. Cells recognize and respond to RNA virus infection through several nucleic acid sensors, including Toll-like receptor 3 (TLR3) and the cytoplasmic double-stranded RNA (dsRNA) sensors retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (10, 83, 200, 201). Binding of RNA to these pathogen recognition receptors results in downstream activation of transcription factors such as IFN regulatory factor 3 (IRF3) and IRF7 and expression of IFN-stimulated genes. An emerging literature suggests that RIG-I and IRF3 have essential functions in responding to WNV infection. Murine embryonic fibroblasts deficient in RIG-I demonstrated delayed induction of host responses, decreased IRF3 activation, and augmented viral replication (56, 57). In contrast, MDA5 may be less essential for cellular recognition of WNV, as IFN production by MDA5-deficient dendritic cells remained largely intact after WNV infection (60). Unlike RIG-I and MDA5, TLR3 is expressed primarily in endosomes and activates IRF3 downstream of the kinases TBK1 and IKKε (54, 124, 169). Although WNV appears to interfere with poly(I-C)-induced IFN responses (164), initial studies suggest that TLR3 is somewhat dispensable for recognition of WNV in some cell types in vitro (56). $TLR3^{-/-}$ mice paradoxically showed decreased lethality after intraperitoneal infection, presumably because of blunted cytokine responses (e.g., TNF-α), which

TABLE 1. WNV infection in immunodeficient mice after peripheral infection

Immune function and mouse strain[a]	Viral strain (lineage)	% Lethality	Virologic and immune characterization[b]	Reference(s)
WT				
C57BL/6	NY2000 (I)	30–35	V—LN, S, SPL, CNS; I—innate, T, B	46, 47, 93, 125, 126, 161, 171, 173, 174
129 Sv/Ev	NY2000 (I)	60	V—LN, S, SPL, CNS; I—innate, T, B	126, 160
C57BL/6 × 129 Sv/Ev	NY2000 (I)	50	V—LN, S, SPL, CNS; I—B	126
BALB/C (5–6 wk)	NY99 NY2000 (I)	80	V—S, SPL, CNS, KD, HT	97, 170
ICR	WN25 (I)	0	V—none in S or CNS	72
IFN				
IFN-α/βR$^{-/-}$ (129 Sv/Ev)	NY2000 (I)	100	V—↑ in all tissues	160
IFN-γ$^{-/-}$ or IFN-γR$^{-/-}$	NY2000 (I)	95	V—↑ in S, LN, SPL, early CNS entry; I—IFN-γ^{+} γδ T cells, ↓ viral loads	174
IFN-γ$^{-/-}$	2741 (I)	90	No data reported	185
RNase L$^{-/-}$	NY2000 (I)	51	V—↑ in LN and SPL	161
PKR$^{-/-}$ × RNase L$^{-/-}$	NY2000 (I)	90	V—↑ in LN and SPL, early CNS entry	161
Complement				
C3$^{-/-}$ (C57BL/6 × 129 Sv/Ev)	NY2000 (I)	100	V—↑ in CNS; I—↓ IgM and IgG	126
CR1/CR2$^{-/-}$	NY2000 (I)	90	V—↑ in CNS; I—↓ IgM and IgG	126
C4$^{-/-}$	NY2000 (I)	100	V—↓ in SPL, ↑ in CNS; I—↓ IgM and IgG; ↓ T-cell responses	125
C1q$^{-/-}$	NY2000 (I)	83	V—no SPL infection, ↑ in CNS; I—delayed IgG production	125
fB$^{-/-}$	NY2000 (I)	96	V—↑ in SPL and CNS, early CNS entry; I—↓ T-cell response	125
fD$^{-/-}$	NY2000 (I)	74	V—↑ in SPL and CNS, early CNS entry	125
C1q$^{-/-}$ × fD$^{-/-}$	NY2000 (I)	90	No data reported	125
C5aR$^{-/-}$	NY2000 (I)	40	No Δ in lethality	125
Other innate immunity effectors				
Flv resistance gene carriers	IS-98-ST1(I)	0	V—↓ in CNS, decreased viral spread	122
B-cell function				
μMT (B cell deficient)	NY2000 (I)	100	V—↑ in S, SPL, and CNS; I—protected by immune serum	46
Secretory IgM$^{-/-}$	NY2000 (I)	100	V—↑ in S and CNS; I—↓ IgG responses, protected by transfer of immune IgM	47
T-cell function				
CD8 α chain$^{-/-}$	NY2000 (I)	84	V—↑ in CNS, ↓ clearance in SPL, early CNS infection, persistent virus in brain	171
TCR β chain$^{-/-}$	2741 (I)	90	V—↑ in SPL and brain	185
β2-Microglobulin$^{-/-}$	Sarafend (II)	80	V—early CNS infection↑ in brain	187
MHC-Ia$^{-/-}$	NY2000 (I)	88	V—persistent virus in brain	171
Perforin$^{-/-}$	NY2000 (I)	78	V—↑ in SPL and CNS, persistent virus in brain; I—transfer of WT CD8^{+} T cells ↓ viral load	173
Perforin$^{-/-}$	Sarafend (II)	18	No Δ in lethality	188
Granzyme A and B$^{-/-}$	Sarafend (II)	70	V—↑ in CNS	188
Perforin$^{-/-}$ × granzyme A and B$^{-/-}$	Sarafend (II)	50	V—↑ in CNS	188
Fas$^{-/-}$	Sarafend (II)	32	No Δ in lethality	188
Gld (Fas ligand$^{-/-}$)	Sarafend (II)	35	No Δ in lethality	188
Perforin$^{-/-}$ × Gld	Sarafend (II)	62	V—↑ in CNS	188
TCR δ$^{-/-}$ (γδ T cell deficient)	2741 (I)	100	V—↑ in S, SPL, and CNS, early spread to CNS; I—protected by transfer of IFN-γ-sufficient γδ T cells, role in priming adaptive immunity	184, 185
Chemokines and chemokine receptors				
CXCL10$^{-/-}$	NY2000 (I)	90	V—↑ in CNS; I—↓ CD8^{+} and CD4^{+} T-cell trafficking to CNS	93
CCR5$^{-/-}$	NY1999 (I)	100	V—↑ in CNS; I—↓ leukocyte trafficking to CNS, transfer of WT CD8^{+} T cells improved survival	61
CCR1$^{-/-}$	NY1999 (I)	~40	No Δ in lethality	61
CX3CR1$^{-/-}$	NY1999 (I)	~40	No Δ in lethality	61
Multiple deficiencies				
RAG (B and T cell deficient)	NY2000 (I)	100	V—↑ in S, SPL, and CNS	46
SCID (ICR)	WN25 (I)	ND	V—persistent viremia and ↑ in brain	72
Cell depletion studies				
Myeloid cells	WN25 (I)	75	V—↑ in S and entry into CNS	15
NK cells	NY2000 (I)	~35	No Δ in lethality	173
CD8^{+} T cells	Sarafend (II)	70	V—↑ in brain	187

[a] C57BL/6 unless otherwise noted.
[b] V, virologic; I, immune; LN, lymph node; S, serum; SPL, spleen; KD, kidney; HT, heart; ↑, increase; ↓, decrease; Δ, change; T, T cell; B, B cell.

apparently facilitate WNV entry into the CNS (186). Nonetheless, it remains possible that TLR3 contributes to WNV recognition and IFN induction in specific immune cell populations, such as macrophages and dendritic cells.

Activation of pathogen recognition receptors stimulates IFN production and feedback amplification of the IFN-stimulated gene response. Studies have begun to elucidate the specific antiviral effector molecules that control WNV infection. Recent reports indicate that the dsRNA-dependent protein kinase (PKR) and 2′-5′-oligoadenylate synthase (OAS) proteins mediate intracellular resistance to WNV. PKR is activated by binding dsRNA and phosphorylates eukaryotic translation initiation factor 2α, resulting in attenuation of protein synthesis (127). RNase L is activated by 2′-5′-linked oligoadenylates synthesized by OAS enzymes and functions as an endoribonuclease that cleaves viral and host RNAs (175). RNase $L^{-/-}$ murine embryonic fibroblasts and $PKR^{-/-} \times$ RNase $L^{-/-}$ bone marrow-derived macrophages supported increased WNV replication in vitro (161, 162). Moreover, mice deficient in both PKR and RNase L showed increased lethality following WNV infection, with greater viral loads in peripheral tissues at early time points after infection (161). Flavivirus susceptibility in mice has been mapped to a mutation in the *Oas1b* gene that results in the expression of a truncated OAS isoform (122, 146). However, the mechanisms by which *Oas* gene alleles affect flavivirus pathogenesis remain uncertain; recent reports suggest that *Oas1b* gene effects on WNV replication are independent of RNase L (161, 162).

Complement. The complement system is a family of serum proteins and cell surface molecules that participate in pathogen recognition and clearance (158). Complement activation occurs through the classical, lectin, and alternative pathways, which are initiated by binding of C1q or mannan-binding lectins or through spontaneous hydrolysis of C3, respectively. Complement contributes to host protection through direct opsonization and/or cytolysis, chemotaxis, immune clearance, and modulation of B- and T-cell functions (29). Complement is required for protection from lethal WNV infection in mice. WNV activates complement in vivo, and mice lacking in the central complement component C3 or complement receptors 1 and 2 showed enhanced lethality after WNV infection (125, 126). All three pathways of complement activation are important for controlling WNV, as mice deficient in alternative, classical, or lectin pathway molecules exhibited increased mortality. Interestingly, the activation pathways modulated WNV infection through distinct mechanisms. Alternative-pathway-deficient mice demonstrated normal B-cell function but impaired $CD8^+$ T-cell responses, whereas classical-pathway- and lectin pathway-deficient mice had defects in both WNV-specific antibody production and T-cell responsiveness (125).

Cellular innate immunity. While few studies have directly addressed the function of cellular innate immunity in WNV infection, limited data suggest that macrophages and dendritic cells may directly inhibit WNV. Macrophage uptake of WNV could control infection through direct viral clearance, enhanced antigen presentation, and cytokine and chemokine secretion. Consistent with this, depletion of myeloid cells in mice enhanced lethality after WNV infection (15). Macrophages may also control flaviviruses through the production of nitric oxide intermediates (99, 112), although the role of nitric oxide in WNV infection has not been established. Less is known about the specific protective roles of dendritic cells in WNV infection, although it is likely that they produce IFN-α/β soon after infection and function as antigen-presenting cells to prime the adaptive immune response.

γδ T cells also function in early immune responses and directly limit WNV infection. As they lack classical MHC restriction, γδ T cells can react with viral antigens in the absence of conventional antigen processing (28, 176). γδ T cells expand following WNV infection in wild-type mice, and increases in viral burden and mortality were observed in mice deficient in γδ T cells (185). Bone marrow chimera reconstitution experiments demonstrated that γδ T cells require IFN-γ to limit WNV infection (174). NK cells also have the potential to control WNV infection through recognition and elimination of virus-infected cells. NK cell activity was transiently activated and then suppressed following flavivirus infection in mice, suggesting that these viruses may have developed a mechanism to inhibit NK cell responses (180). Some viruses evade natural killing by increasing surface expression of MHC or MHC-like molecules (77, 89, 90). Although it is not clear whether WNV utilizes this strategy in vivo, WNV infection in vitro increases surface MHC-I expression in some cell types by enhancing the activity of the transporter associated with antigen processing and by NF-κB-dependent transcriptional activation of MHC class I genes (33, 48, 91, 117). Notably, antibody depletion of NK cells in mice did not alter morbidity or mortality after WNV infection (173), and similar results were obtained by using Ly49A transgenic mice that lack functional circulating NK cells (88) (M. Engle and M. Diamond, unpublished results).

ADAPTIVE IMMUNE RESPONSES TO WNV INFECTION

Humoral responses. Humoral immunity is an essential aspect of immune system-mediated protection from WNV (16, 27, 46, 47, 139, 177, 183). B-cell-deficient mice uniformly died after WNV infection but were protected by passive transfer of immune sera (46). Soluble IgM is required, as $IgM^{-/-}$ mice developed high viral loads in all tested tissues and demonstrated complete lethality after WNV infection (47). In prospective studies, the level of WNV-specific IgM at day 4 after infection predicted disease outcome. While it is apparent from passive-transfer studies that immune IgG can protect against flavivirus infection, the function of IgG during primary infection is less clear. In mice, WNV-specific IgG is not produced until somewhat late in infection (days 6 to 8), after both viral seeding of the CNS and clearance from peripheral tissues have occurred (46, 171). Thus, while it is possible that WNV-specific IgG alters WNV infection of the CNS, current data suggest that by the time IgG is produced, the survival of the animal may already have been largely determined. Additional studies are required to more clearly establish how IgG modulates WNV pathogenesis during primary infection.

Mechanisms of antibody protection. The majority of neutralizing antibodies are directed against regions of the WNV E protein, although a subset likely recognizes the prM protein (40, 52, 150, 181). The E protein has three structural domains that mediate viral attachment, entry, and viral assembly and elicit antibodies with distinct neutralization potentials. Domain

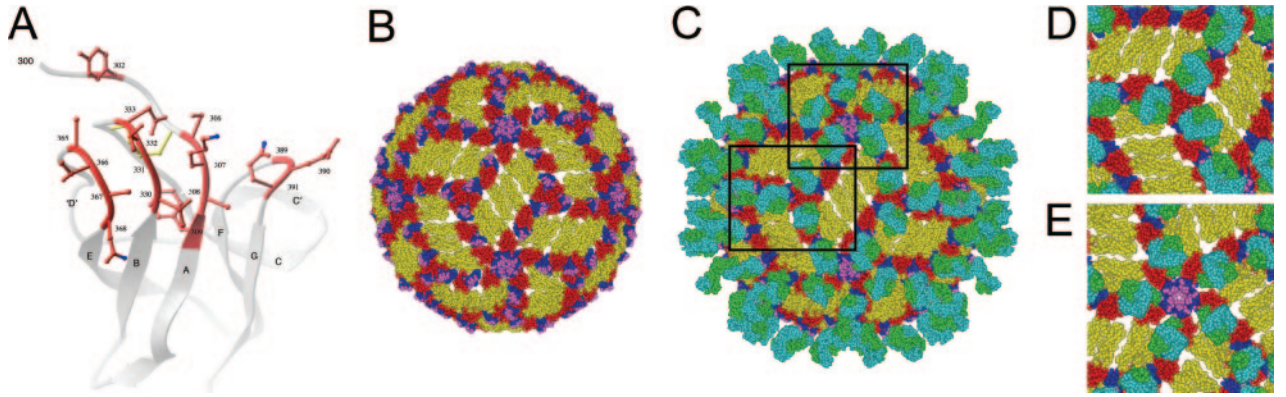

FIG. 1. E16 contact residues and binding of WNV virions. (A) E16 contact residues (red) on DIII of the WNV envelope protein are located in the amino terminus (residues 302 to 309) and three strand-connecting loops, BC (residues 330 to 333), DE (residues 365 to 368), and FG (residues 389 to 391). (B) Pseudoatomic model of the cryoelectron microscopic reconstruction of the WNV virion. The E16 structural epitope is mapped in magenta. (C) Saturation binding of E16 on the WNV particle. E16 is predicted to bind 120 out of 180 potential epitopes with exclusion from the inner fivefold axis. (D and E) Magnified regions of the boxed areas in panel C. This figure is reprinted with permission from Macmillan Publishers Ltd. (Nature **437:**764–768, copyright 2005).

III (DIII) contains the putative receptor binding domain, DII encodes the fusion loop involved in pH-dependent fusion of virus and host cell membranes, and DI participates in E-protein structural rearrangements required for fusion (1, 18, 38, 128). Crystallography, nuclear magnetic resonance, and epitope-mapping studies have shown that E-specific neutralizing antibodies map to all three domains (11, 12, 31, 42, 109, 139, 156, 182, 193). Human single-chain variable-region antibody fragments against DII were protective in mice when administered prior to and after WNV infection (66). However, the most potent inhibitory antibodies recognize a dominant neutralizing epitope on the lateral face of DIII (12, 139, 182). Neutralizing antibodies to this DIII epitope were detected in serum from WNV-convalescent patients and protected mice even when administered after WNV had spread to the CNS. Recent data suggest that DIII-specific antibodies may be particularly potent inhibitors because they block at a postattachment stage, possibly by inhibiting viral fusion (64, 137). The structural basis of DIII-specific antibody neutralization has been investigated by crystallographic analysis of DIII in complex with the Fab fragments of a neutralizing monoclonal antibody (137). These studies demonstrated that the neutralizing antibody E16 engaged 16 residues in four discontinuous DIII regions that localize to the amino terminus (residues 302 to 309) and three strand-connecting loops (residues 330 to 333, 365 to 368, and 389 to 391). Additional crystallographic modeling and cryoelectron microscopy studies have shown that only 120 of the available 180 E-protein epitopes are occupied by E16, suggesting that epitope saturation is not required for neutralization (83a, 137) (Fig. 1).

Although neutralizing antibodies generated during WNV infection predominantly bind structural proteins, antibodies to the nonstructural protein NS1 also protect mice against WNV infection (39). NS1 is a highly conserved glycoprotein that is not packaged within the virus but is secreted at high levels from infected cells and associates with cell surface membranes through undefined mechanisms (118, 123, 191, 192). NS1 is a cofactor in replication and is detected in the serum of infected animals during the acute phase of WNV disease (32, 118). Although the function of soluble NS1 in WNV pathogenesis is not well understood, a recent study suggests that NS1 may inhibit complement activation by binding regulatory protein factor H (K. M. Chung, K. Liszewski, G. Nybakken, R. R. Townsend, D. Fremont, J. P. Atkinson, and M. S. Diamond, submitted for publication). Competitive-binding assays and yeast surface display mapping studies demonstrated that antibodies against WNV NS1 localize to at least three discrete regions of the protein (39). Individual NS1 antibodies protected mice from lethal WNV infection through distinct mechanisms, as some required Fc-γ receptors whereas others did not. Thus, while significant progress has been made in understanding the mechanisms of protection by passive antibody transfer, further studies are needed to define the epitope specificity and function of antibodies that develop during the course of WNV infection.

T-cell responses during primary infection. Experiments with small-animal models have demonstrated that T lymphocytes are an essential component of protection against WNV (27, 84, 116, 136, 171, 173, 187). Consistent with this, individuals with hematologic malignancies and impaired T-cell function have an increased risk of neuroinvasive WNV infection (134, 152). Upon recognition of a WNV-infected cell that expresses class I MHC molecules, antigen-restricted cytotoxic T lymphocytes proliferate, release proinflammatory cytokines (48, 84, 100), and lyse cells directly through the delivery of perforin and granzymes A and B or via Fas-Fas ligand interactions (74, 159). Mice deficient in $CD8^+$ T cells or class I MHC molecules had normal humoral responses but greater and sustained WNV burdens in the spleen and CNS and increased mortality (171, 187). Granzymes appear to be important for control of the lineage II isolate Sarafend, with perforin, Fas, and Fas ligand having a more limited role in modulating infection (188). In contrast, $CD8^+$ T cells require perforin to control lineage I WNV as mice deficient in perforin molecules had increased CNS viral burdens and lethality (173). Moreover, adoptive transfer of wild-type but not perforin-deficient $CD8^+$ T cells decreased CNS viral burdens and enhanced survival. $CD4^+$ T cells likely contribute to the control of infection through mul-

tiple mechanisms, including $CD8^+$ T-cell priming, cytokine production, B-cell activation and priming, and direct cytotoxicity. Preliminary data suggest that $CD4^+$ T cells restrict WNV pathogenesis in vivo. $CD4^+$ T-cell depletion or a genetic deficiency in MHC class II molecules results in attenuated WNV-specific antibody responses and increased lethality (E. Sitati and M. Diamond, unpublished data).

Memory responses. Although much remains to be learned about the specific mechanisms that define immunity against WNV, both humoral and cellular responses likely are essential for protection. Specific neutralizing antibodies are generated at late times after primary WNV infection (46, 139, 141, 177), and the development of high-titer neutralizing antibodies after vaccination correlates with protection against challenge (5, 106, 177). Nevertheless, it remains unknown how the epitope specificity of the antibody repertoire contributes to protective memory responses. T-cell-mediated immunity is also likely necessary to resist a second WNV challenge. Recent studies suggest that γδ T cells contribute directly to priming protective adaptive immune responses to WNV infection in mice (184). γδ T-cell-deficient mice infected with WNV had decreased memory $CD8^+$ T-cell responses and were more susceptible to secondary infection, and adoptive transfer of $CD8^+$ T cells from WNV-primed wild-type but not T-cell receptor (TCR) $\delta^{-/-}$ mice increased the survival of naive mice. Immunization with different WNV vaccine preparations can induce memory T cells (4, 130, 197), although the relative contribution of humoral versus cellular memory to vaccine efficacy remains largely undefined.

CNS IMMUNE RESPONSES TO WNV

Since WNV is naturally acquired through peripheral inoculation and then spreads to the CNS, the immune response must control viral replication in both compartments. The CNS represents a challenge to the immune system, as effective resolution of CNS infection requires clearance with limited damage to critical nonrenewing cells such as neurons (68). Several features of the innate and adaptive immune response mitigate WNV pathogenesis in the CNS. IFN-α/β controls WNV replication in neurons and has an independent role in modulating survival. Mice that lack the IFN-α/βR had increased viral loads and died earlier following intracranial WNV inoculation (160). Recent data suggest that IFN-α/β induces cell-specific antiviral programs in different neuronal populations: PKR and RNase L mediated the antiviral effects of IFN in cortical neurons after WNV infection, but these molecules were dispensable for protection in peripheral neurons (161). For some encephalitic viruses, IFN-γ can mediate noncytolytic viral clearance from infected neurons in vitro and in vivo (19, 24, 155); however, control of WNV infection in the CNS appears to be largely independent of IFN-γ. Mice deficient in IFN-γ did not demonstrate delayed viral clearance from the CNS or enhanced lethality after intracranial infection (174).

T-cell-mediated immunity is essential for controlling WNV infection in the CNS. $CD8^+$ T cells traffic to the brain after WNV infection in mice, and their presence correlates temporally with viral clearance (126, 171, 173, 187). $CD8^+$ T $cell^{-/-}$, MHC class $I^{-/-}$, and $perforin^{-/-}$ mice all showed WNV persistence in the brain, with detectable infectious virus up to 35 days postinfection (171, 173). Similarly, mice deficient in MHC class II molecules showed persistent WNV infection in the brain and spinal cord up to 40 days postinfection (Sitati and Diamond, unpublished). Thus, the absence of functional

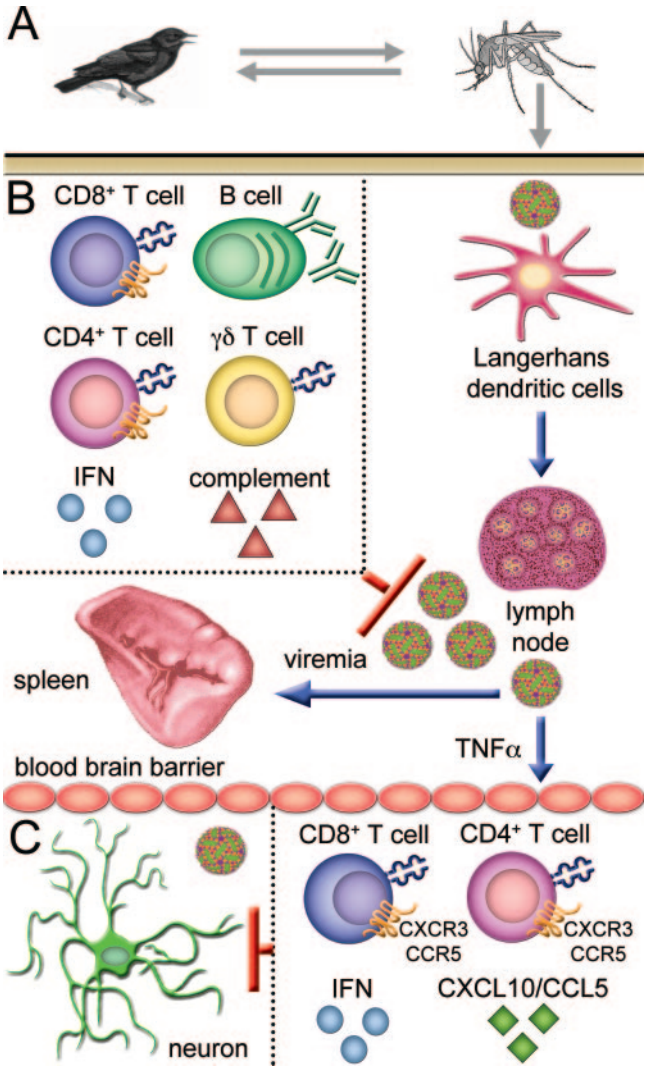

FIG. 2. WNV dissemination and immune system control. (A) WNV is maintained in nature in an enzootic mosquito-bird-mosquito transmission cycle. (B) Following *Culex* mosquito inoculation, WNV replicates in skin Langerhans dendritic cells, which traffic the virus to the lymph node, where further replication ensues. Following induction of a primary viremia, WNV spreads to other peripheral organs. Several aspects of the innate and adaptive immune response limit WNV replication in the periphery. IFN-α/β acts as an antiviral agent that restricts viral translation and replication soon after infection. B cells and antibody (primarily IgM) modulate viral levels in serum and prevent early CNS seeding, while complement is required for efficient priming of humoral and cellular immune responses. IFN-γ-secreting γδ T cells control viral replication through direct antiviral mechanisms and contribute to the generation of adaptive immune responses. $CD4^+$ and $CD8^+$ T cells participate in viral clearance from peripheral tissues. (C) Following replication in the periphery, WNV spreads to the CNS possibly through TNF-α-mediated changes in BBB permeability. Neurons are the primary target of WNV in the brain and spinal cord. IFN-α/β is required to control WNV infection in the CNS and may prolong neuronal survival. The chemokines CXCL10 and CCL5 and their cognate ligands CXCR3 and CCR5 aid in recruiting $CD4^+$ and $CD8^+$ T cells and monocytes to the CNS, where they function to clear virus from infected tissues.

TABLE 2. WNV virulence factors

Trait	Source	Effect on pathogenesis	Reference(s)
Diverse cellular and species tropism	Use of multiple and/or well-conserved receptors	Replication in multiple tissues and diverse clinical manifestations	46, 53, 58, 79, 96, 157, 195
Induction of rapid cell death	NS3, capsid	May contribute to neuropathology	145, 153, 172, 198
N-linked glycosylation of E protein	Genetic variation	Alters virion stability, replication, particle assembly, pathogenesis	14, 73, 107, 170
N-linked glycosylation of NS1 protein	Genetic variation	Lack of glycosylation reduces pathogenesis in vivo	Whiteman et al., submitted
IFN resistance	NS2A, NS2B, NS3, NS4A, NS4B, genetic variation	May suppress IFN production in vivo and allow increased replication or pathogenesis	71, 114, 115, 132
Quasispecies generation	Low-fidelity RNA-dependent RNA polymerase	Potential for escape from antibody neutralization and T-cell lysis	12, 80, 108
Upregulation of MHC class I expression	Dependent on NF-κB activation	May inhibit NK cell responses	33, 48, 85, 90, 117

$CD8^+$ or $CD4^+$ T cells results in failure to clear WNV from infected neurons in the CNS. Since the CNS experiences limited immune surveillance in the absence of inflammation, chemokine-dependent T-cell recruitment to infected CNS tissues modulates viral pathogenesis. Following viral infection of the CNS, inflammatory chemokines (e.g., CCL5 and RANTES) are expressed by trafficking leukocytes and resident astrocytes and microglia (6, 103, 143, 154). Surprisingly, WNV infection induced expression of the chemokine CXCL10 in neurons, which recruited effector $CD8^+$ T cells through its cognate ligand, CXCR3 (93). A genetic deficiency in CXCL10 resulted in reduced T-cell trafficking to the CNS, greater viral loads in the brain, and enhanced mortality. The chemokine receptor CCR5 also regulates T-cell trafficking to the brain during WNV infection; its absence resulted in depressed CNS leukocyte migration and increased lethality in mice (61). Collectively, these experiments suggest that multiple aspects of the innate and adaptive immune responses function together to control WNV infection in the periphery and in the CNS (Fig. 2).

VIRAL AND HOST FACTORS THAT MODULATE DISEASE OUTCOME

Molecular basis of virulence. The ability of WNV to survive and cause disease within the host depends on its capacity to infect target cells and evade immune system recognition (Table 2). Certain aspects of the biology of WNV facilitate its ability to cause severe disease. WNV productively infects diverse cell populations from many animal species, suggesting usage of multiple and/or well-conserved receptors (9, 53, 58, 79, 157, 178, 195). The relatively diverse tropism of WNV allows viral replication in several tissues in animal and human hosts and may contribute to the wide spectrum of clinical manifestations (76, 142, 167, 199). WNV is cytolytic and induces apoptosis in a variety of cell types, including neurons (145, 172). Although few studies have investigated the mechanisms of WNV-induced cell death in vivo, individual WNV proteins may contribute to virus-mediated cytotoxicity. In vitro, expression of either NS3 or capsid protein induced rapid, caspase-dependent apoptosis, and capsid protein expression in vivo resulted in cell death (153, 198).

Genetic variation also affects WNV virulence. Sequence-based phylogenic analyses have defined two genetic lineages of WNV, lineages I and II (101, 163). Lineage I strains are detected worldwide and have been responsible for recent lethal human outbreaks, including those in Romania (1996), Russia (1999), Israel (1998 to 2000), and the Americas (1999 to 2005) (43, 120). In contrast, lineage II strains appear to be localized to central and southern Africa and have caused occasional human infections (82, 101). In birds and mammals, lineage I strains generally induce significant encephalitis and mortality, although isolates from both lineages can be neuroinvasive (13, 21, 46, 104, 195). Thus, while lineage I WNV isolates appear to be linked to the recent increase in severe infection of humans, pathogenic lineage II isolates have been identified and have the potential to induce significant human disease. The specific sequence determinants of virulence are an area of intensive study. N-linked glycosylation of the E protein appears important for neuroinvasion as mutations of E-protein glycosylation sites attenuated viral replication and pathogenesis (13, 14, 170). Moreover, E-protein glycosylation modulates WNV virulence by altering virion stability, viral replication, and particle assembly (14, 73, 107). Glycosylation of the NS1 protein has also been linked to WNV pathogenesis. WNV NS1 contains three N-linked glycosylation sites (residues 130, 175, and 203); mutants lacking glycosylation at either two or three sites induced lower viremia and decreased lethality in vivo (M. C. Whiteman, R. M. Kinney, D. W. C. Beasley, K. M. Chung, M. S. Diamond, T. Solomon, and A. D. T. Barrett, submitted for publication).

WNV has also evolved specific strategies to avoid and/or attenuate innate and adaptive immune responses. Flaviviruses, including WNV, are largely resistant to the antiviral effects of IFN once cellular infection is established. Through studies with WNV, Japanese encephalitis virus, dengue virus, yellow fever virus, and Langat virus, this phenotype has been largely ascribed to the actions of nonstructural proteins NS2A, NS2B, NS3, NS4A, NS4B, and NS5. These nonstructural proteins disenable IFN-induced responses at multiple stages within the cell by delaying IRF-3 activation and IFN-β gene transcription and by impairing JAK1 and Tyk2 phosphorylation (17, 71, 110, 111, 115, 132, 133). The replication fitness and virulence of lineage I and lineage II strains has been linked to control of host IFN responses (83b). A pathogenic lineage I Texas isolate

actively antagonized IFN signaling, whereas an attenuated lineage II strain from Madagascar lacked this activity. The replication and virulence of the lineage II isolate were restored in cells and mice that lacked the IFN-α/βR. These data suggest that inhibition of type I IFN responses may be a key feature in the evolution of pathogenic WNV strains. Consistent with this, a mutation in the NS2A protein of Kunjin virus resulted in increased IFN production in weanling mice and attenuated neurovirulence (114). Escape from the humoral immune response may also contribute to WNV pathogenesis. Flaviviruses have a low-fidelity RNA-dependent RNA polymerase that generates quasispecies in vivo (80). This antigenic variation may allow viral quasispecies to escape antibody-mediated neutralization (12), as strains with mutations at the dominant neutralizing epitope in DIII of the E protein can emerge (108).

Host genetic determinates of WNV susceptibility. A limited number of genetic risk factors have been correlated with increased susceptibility to WNV. In inbred mouse strains, susceptibility to infection by flaviviruses, including WNV, has been mapped to a truncated isoform of the gene for OAS1b (122, 146). Although an analogous deletion has not been linked to severe WNV infection in humans, a recent study with 33 WNV-infected patients showed an increased frequency of an OAS splice-enhancer site that could result in a dominant-negative protein (196). Another study examined *CCR5Δ32*, a relatively common mutant allele of the chemokine receptor CCR5, in WNV-infected cohorts (62). A greater incidence of *CCR5Δ32* homozygosity was observed in symptomatic and lethal WNV cases, suggesting that CCR5 may mediate resistance to WNV infection in humans.

FUTURE PERSPECTIVES

The use of animal models has fostered a significant understanding of the balance between WNV pathogenesis and immune system-mediated control. These studies establish that innate immunity, humoral immunity, and T-cell-mediated immunity are all required to orchestrate effective control of WNV. The absence of one or more of these factors often results in fulminant and fatal infection. To modulate these host defenses, WNV has developed countermeasures that attenuate innate and adaptive immune responses and facilitate its infectivity in vivo. Several important questions regarding WNV infection and host interaction remain unanswered. Although WNV induces lethal encephalitis, the exact mechanisms by which WNV crosses the BBB, spreads within the CNS, and causes neuronal dysfunction and death require further study. Additionally, although antibodies can protect against WNV infection, much remains to be learned about the specific epitopes and mechanisms that define neutralization. Finally, few studies have characterized which aspects of the memory response to WNV mediate protection. Enhanced investigation of the immunologic basis of protection may facilitate the design of improved vaccines and more-targeted immunotherapies. Such efforts also will likely enhance our understanding of the pathogenesis of related encephalitic flaviviruses that cause human disease.

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